tRNA genes for efficient gene expression in *E.coli* may be a frequent phenomenon for genes derived from organisms possessing a distinct codon usage from that of *E. coli*.--

- At page 7, after line 21, insert the following:

--Figure 2 displays a Coomassie blue-stained SDS PAGE gel containing samples from cells containing a T7-driven expression vector for wild type human cardiac troponin T (hcTnt wt; *argU*-dependent; Hu et al.) and two mutants thereof (mut1, mut2), before and after IPTG treatment and in the presence and absence of an expression vector for the tRNA genes *argU* and *glyU* (RG). The position of the TnT protein is shown with an arrow.--

REMARKS

Claims 1-16 and 18-44 are pending.

Objection to the Specification

With respect to deleting reference to Tables 1-4 in the specification, Applicant submits that the amendments to the specification directed herein removing the reference to Tables 1-4 is sufficient to overcome this objection. The amendment adds no new matter because the scope of the invention is not affected by the deletion. The codon usage information contained in the canceled tables was known in the art at the time of filing of the application. For example, Table 1 present in the 60/117,355 priority document states on its face that the information on codon usage was compiled from information then available in a review article (Kane, 1995, Current Opinion in Biotechnology 6: 494-500) and on the codon usage World Wide Web site http:\\www.dna.affrc.go.jp/~nakamura/codon.html. Tables 2-4 of the priority document state on their face that they were compiled from the same codon usage Web site, showing that the information was known and available as of the January 27, 1999 filing date of the priority document. Therefore, it was not necessary to include the information contained in the tables in the specification, and one skilled in the art could practice the invention given the specification and the prior art codon usage information alone.

The specification was also objected to because there is no description of Figure 2 in the Brief Description of the Drawings and the references to the figures made in the specification

appear to be consistently drawn to the wrong figure. Applicant submits that the amendment entering a description of Figure 2 as it appears in the application and the amendments changing numbers used in the specification to refer to the figures are sufficient to overcome this objection. The amendments add no new matter.

Rejection under 35 U.S.C. §112, first paragraph

All pending claims are rejected under 35 U.S.C. §112, first paragraph as "containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention." The Office Action states that "the claimed invention literally encompasses a combination of any gene coding sequence comprising codons which might be rarely used in a host cell, any host cell derived from any source and any tRNA gene encoding a tRNA corresponding to a rarely used codon" (emphasis in original). The Office Action further states that "while the specification provides a broad list of proposed cell types for practicing the claimed invention (e.g., various plant and animal cell types, various fungal cell types, etc.), the actual description and relevant working examples provided in the specification for combinations of rarely used codons/host cell type/tRNA genes are limited to expression of polypeptides in E. coli." Finally, the Office action states that there are no codon frequency data provided in the specification for other than E. coli, and that "no tRNA genes corresponding to such rarely used codons for other cell types are provided in the specification." From this, the Office Action concludes that one of skill in the art would reasonably conclude Applicant was not in possession of the claimed invention. Applicant respectfully disagrees.

First, Applicant submits that the specification and the claims as filed clearly set forth the invention in a manner such that one skilled in the art is on notice that Applicant was in possession of the full scope of the claimed invention as of the filing date of the application. Written description for a genus claim requires that a representative number of embodiments of the claimed genus be described such that one skilled in the art can envision the whole genus. Applicant submits that the specification contains description of such embodiments, as discussed below.

The specification states that "Suitable host cells for transfection with a set or array of tRNA genes and expressing the DNA encoding the desired polypeptide are the prokaryotic, yeast, or higher eukaryote cells." (page 16, lines 25-26). The fact that specific codon usage will vary between any two species of organisms, whether from the same or different kingdoms, does not mean that one skilled in the art cannot envision the full range of embodiments encompassed by the invention from the description provided in the specification and claims as filed. The specification goes on to describe numerous host cells in each of the prokaryotic (over 20 species; page 16, line 27 to page 18, line 12), yeast or fungi (20 species; page 18, line 14 to page 19, line 5), and higher eukaryotic systems, including insects (6 species described; page 19, lines 10-19), plants (7 species described; page 19, lines 20-21), and mammalian host cells (17 specific cell lines described from mouse to man; lines 4-15). This is in addition to the written description for the working examples of the practice of the invention in prokaryotic host cells. Thus, the genus of host cells encompassed by the claims is described in a manner sufficient to satisfy the written description requirement of §112, first paragraph.

The Office Action provides no reason to believe that the invention could not be practiced in any cell type that is transfectable, where host and exogenous gene codon usages are known. Those skilled in the art know whether a given host cell is transfectable; indeed, the very designation "host cell" implies that there must be some way known in the art to introduce exogenous genetic material. Transfection methods for bacteria are well known in the art, and the specification describes methods for transfection of host cells by appropriate means, including calcium phosphate, electroporation, calcium chloride, infection with Agrobacterium tumefaciens, ultrasound, microinjection, protoplast fusion, and methods using polycations (seepage 20, line 20 to page 21, line 18).

The Office Action acknowledges that differences in codon usages are well known in the art. Therefore, specific codon usages need not be described. What must be described, rather, is the way in which specific knowledge of codon usages would be used in the practice of the invention. This is clearly set forth in the specification, examples, and claims as filed. For example, even the Abstract states that

> "High level expression of heterologous proteins in host cells is frequently limited by the presence of rarely utilized codons, due to depletion of the internal tRNA

> pool and stalling of translation. This invention provides expression host cells generated by introduction of a vector for the expression of an array of tRNA genes that are rare in the host cells. The modified host cells are capable of efficiently supporting expression of selected recombinant genes which otherwise would be limited by the presence of rare codons." (Abstract, page 1)

Applicant submits that from this description alone, one skilled in the art would realize that tRNA genes specific for low frequency codons are transfected into host cells to generate cells useful for expression of genes with the low frequency codons. Using information regarding codon usage by the host cell and the gene of interest, an array of three or more tRNA genes can be designed to meet the needs of a high level expression system for a given organism (see page 14, line 25 to page 14, line 8). Not only is the invention described in terms that encompass the genus of host cells covered by the claims, absent any evidence suggesting that the approach would not work in a given transfectable system for which codon usage information is known, the description of the invention should not be seen as limited by the scope of the specific working examples.

Finally, the law does not require that a patent specification teach each and every embodiment falling within a given claim in detail, but only a representative number of species. The specification teaches at least five tRNA genes, argU, ileY, lueW, proL and glyU, corresponding to rarely used codons. Numerous tRNA genes were known in the prior art for numerous species before the filing date of the application. A search of GenBank for just one tRNA, tRNA-Gly, limited to exclude tRNA synthase hits, resulted in 1192 hits, many for specific tRNA-Gly genes from diverse species, and others for complete or partial genome sequences in which tRNA-Gly genes are identified. This search includes post-filing entries, but Applicant submits that if even, for example, a quarter of those sequences were available in the art before filing (January 27, 1999), which is likely, there would still have been hundreds of tRNA genes for tRNA-Gly known as of the filing date. This is not to mention known tRNA genes specific for codons corresponding to the remaining 19 amino acids. Codon usages were also known in the prior art as of the filing date of the application. One skilled in the art can readily determine whether a tRNA gene sequence with a given codon specificity is available, and knows that many such gene sequences are available. The specification states that host cells can be prokaryotic, yeast or higher eukaryote cells (page 16, lines 25-26). Given this information, one skilled in the art can readily envision and generate a construct that expresses an array of three or more cloned tRNA genes from a given species. The Office Action provides no reason to believe otherwise.

Therefore, the written description requirement is satisfied with regard to tRNA genes from species other than E. coli.

In view of the above, Applicant respectfully requests withdrawal of the §112, first paragraph rejection of claims 1-16 and 18-44.

Rejection under 35 U.S.C. §102(e)

Claims 1-10, 15, 16, 19, 22-27, 32-40 and 42-44 are rejected under 35 U.S.C. §102(e) as being anticipated by Zdanovsky, U.S. Patent No. 6,214,602. Applicant submits that the '602 patent, filed August 28, 1998, is not prior art over the present application. The accompanying Rule 1.131 Declaration of Carsten-Peter Carstens sets forth evidence, in the form of laboratory notebook entries, showing that the presently claimed invention was conceived of and reduced to practice before August 28, 1998.

Specifically, the declaration shows that both conception and reduction to practice of a vector that replicates in a host cell containing a recombinant DNA molecule which comprises an array of three or more tRNA genes, wherein said tRNA genes correspond to codons that are rarely used in said host cell (*argU*, *ileY* and *leuW*), occurred before August 28, 1998. The vector reduced to practice before August 28, 1998 meets the limitations of independent vector claim 22.

The declaration and also shows that both conception and reduction to practice of a host cell (BL21DE3) containing a recombinant DNA molecule which comprises an array of three or more tRNA genes (*argU*, *ileY* and *leuW*), wherein said tRNA genes correspond to codons that are rarely used in said host cell, occurred before August 28, 1998. This host cell meets the limitations of independent host cell claim 1.

Finally, the declaration shows that both conception and reduction to practice of a method according to independent claim 33 occurred before August 28, 1998. The method involved the production of a protein of interest (*Pfu* polymerase), comprising the step of culturing a host cell (BL21DE3-RIL cells) containing a recombinant DNA molecule that comprises an array of three or more tRNA genes, wherein said tRNA genes correspond to codons that are rarely used in said host cell (*argU*, *ileY* and *leuW* wherein said codons are present in the gene for the protein of

interest, and wherein the conditions of culturing said host cell are sufficient to produce said protein of interest.

In view of the above, Applicant submits that the '602 patent is not prior art over the presently claimed invention. Applicant respectfully requests withdrawal of the §102(e) rejection over this reference.

Rejections under 35 U.S.C. §103(a)

Claims 18 and 20-21 are rejected as obvious over the '602 patent in view of Wnendt. Applicant submits that because the '602 patent is not prior art over claim 1, from which claims 18, 20 and 21 depend. Therefore, the combination of Wnendt with the '602 reference cannot render obvious the claimed invention. Applicant respectfully requests the withdrawal of this §103(a) rejection.

Claims 1-5, 10-16, 22, 23 and 26-38 are rejected as obvious over Del Tito et al. in view of Makoff et al. This rejection is maintained from the previous Office Action, paper No. 8, mailed August 9. 2000. The Office Action states that Del Tito et al. teaches the coexpression of an array of two tRNA genes (argU and ileX) encoding tRNAs specific for rarely used codons, along with a gene encoding the heterologous polypeptide, Mup^r IRS, resulting in increased levels of active protein as compared to cells comprising a plasmid only expressing the ileX gene. The Office Action states that the Del Tito et al. reference does not explicitly teach the use of a vector comprising an array of three or more tRNAs corresponding to rarely used codons. The Makoff et al. reference is cited as teaching that expression of tetanus toxin fragment C in E. coli is limited by its high demand for rare tRNA molecules, and that fragment C comprises several different rare codons specifying different amino acids (leu, Ile, Ser, Pro, Arg and Gly). The reference is also cited as teaching that the replacement of the rarely used codons with synthetic sequence that lacks the rarely used codons results in an approximate 4-fold increase in expression of the heterologous polypeptide. The Office Action concludes that it would have been obvious to one of ordinary skill in the art to modify the vector taught by Del Tito et al. comprising argU and ileX by introducing additional tRNA genes corresponding to rarely used codons in order to express the tetanus fragment C subunit in E. coli. Applicant respectfully disagrees.

Claims 6-9, 19, 21, 24 and 25 are rejected under 35 U.S.C. §103(a) as obvious over Del Tito et al. in view of Makoff et al. and the Novagen catalog. This rejection is maintained from the previous Office Action, paper No. 8, mailed August 9. 2000. The Del Tito, and Makoff references are combined in the same way as applied above, with the addition of the Novagen reference offered to show a T7 polymerase-controlled promoter, an IPTG inducible promoter and strains of host bacteria that are protease deficient. Applicant respectfully disagrees with the conclusion as to obviousness over this combination of references.

Claims 18 and 20 are rejected under 35 U.S.C. §103(a) over Del Tito et al. in view of Makoff et al. and the 1997 Novagen catalog, in further view of Wnendt. This rejection is maintained from the previous Office Action, paper No. 8, mailed August 9, 2000. The Del Tito et al. reference is combined with the Makoff et al. and Novagen references in the same manner as in the previous rejections. Wnendt is cited as teaching the use of EndA- strains in order to increase the quality and quantity of plasmid DNAs obtained from host cells. Applicant respectfully disagrees with the conclusion as to obviousness over this combination of references.

Claims 39-44 are rejected under 35 U.S.C. §103(a) as obvious over Del Tito et al. This rejection is maintained from the previous Office Action. Applicant respectfully disagrees with the conclusion as to obviousness over this reference.

With respect to all of the recited 103 rejections, Applicant submits that the claimed invention has met with significant commercial success in the form of sales in the marketplace. Commercial success is an established indicator of nonobviousness and must be taken into account in considering the issue of obviousness. That evidence of such success is "secondary" in time does not mean that it is secondary in importance. Evidence of secondary considerations may often be the most probative and cogent evidence in the record. *Trustwall Systems Corp. v. Hyrdo-Air Engineering Inc.*, 813 F.2d 1207, 2 U.S.P.Q.2d 1034 (Fed. Cir. 1987). It has been held that a showing of commercial success of a claimed invention, whenever such success occurs, is relevant in resolving the issue of nonobviousness. *Lindemann Maschinenfabrik GmbH v. American hoist & Derrick Co.*, 730 F.2d 1452, 1461, 221 U.S.P.Q. 481, 487 (Fed. Cir. 1984).

As an objective indicator of nonobviousness, Applicant submitted evidence of the commercial success of the claimed invention in response to the Office Action mailed August 9, 2000. The present Office Action states that the Rule 132 Declaration of Mary Buchanan, which presented documentation of the commercial success of products embodying the claimed invention, was not persuasive because there is no background against which to judge the degree of success for the claimed invention. The Office Action asks: "Are there any other host strains on the market today for expression of polypeptides comprising rarely used codons? How do sales figures for the embodiments described by Ms. Buchanan compare to such strains? How do the sales figures compare to other expression hosts available on the market?" Further, the Office Action states that "Gross sales figures alone do not show commercial success absent any evidence to market share, the time period during which the product was sold, or as to what sales would normally be expected in the market." The Office Action concludes that "some relevant background figure needs to be provided against which the figures provided in the declaration can be compared." Applicant submits that the Rule 132 Declaration of Mary Buchanan filed in response to the previous Office Action and her supplemental Rule 132 Declaration submitted herewith satisfies the legal requirements with respect to commercial success, as detailed below.

Applicant submits that a patentee asserting commercial success as evidence of non-obviousness must demonstrate a sufficient relationship between the commercial success and the patented invention such that the success can be attributed to the invention. *Alpex Computer Corp. v. Nintendo Co. Ltd.*, 86 Civ. 1749, 34 U.S.P.Q.2d 1167, 1190 (N.Y. 1994); *Ex parte Standish*, 10 U.S.P.Q.2d 1454 (B.P.A.I. 1988). When a patented device is a commercial product, there is an inference that its commercial success is due to the patented device itself, absent a showing to the contrary. *See e.g., Hughes Tool Co. v. Dresser Indus., Inc.*, 816 F.2d 1549, 1556, 2, U.S.P.Q.2d 1396, 1402 (Fed. Cir.), cert denied, 484 U.S. 914 (1987), as interpreted in the unpublished (not citable as precedent) opinion for *Comair Rotron Inc. v. Matsushita Electric Corp. of America*, 33 U.S.P.Q.2d 1785, 1788 (Fed. Cir. 1994)

Applicant submits that the Rule 132 Declaration of Mary Buchanan filed in response to the previous Office Action demonstrated a nexus between the claimed invention and the evidence of commercial success. This evidence establishes that the products sold correspond to embodiments of the claimed invention (see first paragraph under heading 2), and that the

commercial success is directly derived from the claimed invention (see the third paragraph under heading 2). It also establishes that the consumer is free to choose on the basis of objective principles (the sales are not the result of consumption by purchasers normally tied to Stratagene), and that such commercial success is not merely the result of heavy promotion or advertising (see heading 3).

In the instant application, there is no other product on the market during the period for which the strong sales figures are presented. This is established in the Supplemental Rule 132 Declaration of Mary Buchanan that accompanies this response. The lack of any reasonable alternative use for the CodonPlus™ cells, other than for the expression of genes with codon bias, and the higher price of the CodonPlus™ cells relative to general purpose expression hosts makes it so that gross sales figures <u>are, in fact,</u> indicative of commercial success. This commercial success is, in turn, indicative of nonobviousness and cannot be ignored.

The Office Action also states, with regard to the prior declaration of Mary Buchanan, that the strain/vector combinations described in the specification are all drawn towards embodiments wherein the combination of tRNA genes in an array are either argU-ileY-leuW or argU-proL, and that "even if one were to grant that the sales figures provided by the declaration indicate commercial success for these embodiments, it is not clear that such a demand would be present for other claimed embodiments (e.g., ileY-leuW) wherein the tRNA gene combinations may not correspond to a significant number of eukaryotic genes comprising combinations of those rarely used codons." Applicant respectfully disagrees.

It has been held that a patentee need not show that all possible embodiments within the claims were successfully commercialized in order to rely on the success in the marketplace of the embodiment that was commercialized. *Applied Materials Inc. v. Advanced Semiconductor Materials*, 40 U.S.P.Q.2d 1481, 1486 (Fed. Cir. 1996). The question is not whether evidence of commercial success is "commensurate in scope with the claims," but rather whether the evidence is relevant to the question of non-obviousness. *E.I. du Pont de Nemours & Co. v. Phillips Petroleum Co.*, 656 F. Supp. 1343, 2 U.S.P.Q.2d 1545 (Del. 1987). Thus, it is not proper to require commercialization of every claimed embodiment for the evidence of commercial success to be relevant to nonobviousness.

In view of the above, Applicant submits that all patentability issues raised in the Office Action have been addressed and that the claims are in condition for allowance. Applicant respectfully requests such action.

Respectfully submitted,

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Version of amendments marked to show changes:

Paragraph at page 2, lines 7-13:

Expression of heterologous proteins in bacteria such as *E. coli* has become a standard procedure in most molecular biology laboratories and a cornerstone of production in the biotechnology industry. The most frequent problems encountered in bacterial expression systems are the insolubility of the induced protein and the poor efficiency of expression. Expression of heterologous proteins in bacteria can be seriously compromised due to the different codon preference displayed in other organisms. [The codon frequencies in *E.coli* and other organisms are compared in Table 1.]

Paragraph at page 7, line 22 to page 8, line 3:

Figure [2] 3 demonstrates rescue of the expression of a derivative of the CBP-Cre fusion protein by introduction of the *ileY* gene. Cultures of BL21gold DE3 cells containing T7-driven vectors for a CBP/Cre-recombinase fusion gene with either three rare leucine (CBP-3xL-Cre, codon CUA) or isoleucine codons (CBP-3xL-Cre, codon AUA) at the 5' end of the gene, and the indicated pACYC-based tRNA expression vectors were induced at mid-log phase for 1h with 1mM IPTG. Fifteen µl of whole cell lysate were loaded on a 4-20% PAGE gel and stained with Coomassie blue. The position of the induced fusion protein is indicated by an arrow. A sample of CBP-Cre without any extra codons was added as a reference. The lanes labeled with RG contain an expression vector for the tRNA genes *argU* and *glyU*. The lanes labeled RIL contain an expression vector for the *argU, ileY* and *leuW* tRNA genes.

Paragraph on page 8, lines 4-16:

Figure [3] 4 demonstrates the functionality of the *proL* gene in an RILP array. Cultures of BL21goldDE3 strains containing pACYC-based vectors expressing copies of the indicated *E.coli* tRNA genes and the T7-driven expression vectors for human cardiac troponin T (*argU*-dependent) and the Cre-recombinase/CBP fusion genes CBP-exi-Cre (*ileY*-dependent) and CBP-3xP-Cre (*proL*-dependent) were induced for 2h with 1mM IPTG. Fifteen µl of whole cell lysate were loaded on 4-20% PAGE-gels and stained with Coomassie blue. HcTnT is an *argU*-dependent T7-driven recombinant human cardiac troponin T expression construct (Hu et al.).

CBP-3xi-Cre and CBP-3xP-Cre are T7-driven CBP-Cre-recombinase fusion genes containing either three AUA(3xi) codons or three CCC(3xP) codons near the N-terminus of the fusion protein. Cells with the RILP array (containing the *proL* tRNA gene), but not the RIL array, allow efficient expression of the recombinant CBP-3xP-Cre gene. The arrows indicate the position of the recombinant gene products. RIL: *argU, ileY* and *leuW* tRNA genes. RILP: *argU, ileY*, *leuW*, and *proL* tRNA genes.

Paragraph on page 8, lines 17 to 25:

Figure [4] <u>5</u> reveals that the RIL and RILP arrays do not significantly affect the level of expression of well-expressed proteins. The indicated host cells were transformed by T7-driven expression vectors for JNK (human c-jun N-terminal kinase), λ-phosphatase or calmodulin. Cultures were induced at mid-log growth for 2h with 1mM IPTG. Fifteen μl of induced cultures were denatured by boiling in SDS loading buffer, separated on a 4-20% PAGE gel and stained with Coomassie blue. The positions of the induced heterologous proteins are indicated by arrows. "-": BL21gold DE3. RIL: BL21gold DE3 with the *argU, ileY* and *leuW* tRNA gene array. RILP: BL21gold DE3 with the *argU, ileY*, *leuW* and *proL* tRNA gene array.

Paragraph on page 8, line 26 to page 9, line 10:

--Figure [5] <u>6</u> shows that high level expression of *Pfu*-polymerase depends on the presence of extra copies of both the *argU* and *ileY* genes. Cultures of BL21gold DE3 strains containing pACYC-based vectors expressing copies of the indicated *E.coli* tRNA genes and the T7-driven expression vectors for *Pfu*-polymerase, human cardiac troponin T and CBP/Cre-recombinase were induced for 2h with 1mM IPTG. Fifteen μl of whole cell lysate were loaded on 4-20% PAGE-gels and stained with Coomassie blue. High level of expression of human cardiac troponin T (hcTnT) in *E. coli (*expressed from a pET21b construct) is dependent on removal of two tandem AGA/AGG codons or rescue by extra copies of the *argU* tRNA gene. CBP-3xi-Cre is a CBP-tagged Cre-recombinase construct containing 3 extra AUA codons (coding for isoleucine) at the 5' -end of the recombinant gene. High level expression of this protein in *E. coli* is dependent on the presence of extra copies of *E. coli IleY* t-RNA gene (see Fig. 3). RG: pACYC with *argU* and *glyU* tRNA genes. RI: *argU* and *ileY* tRNA genes. IL: *ileY* and *leuW* tRNA genes. RIL: *argU, ileY*, and *leuW* tRNA genes.

Paragraph on page 9, lines 11 to 19:

Figure [6] 7 demonstrates that failure of the RG array to support efficient *Pfu* DNA-polymerase expression is not due to a negative effect of the *glyU* gene. The experiment displayed in Fig. [6] 7 was repeated using an *argU/leuW* array (RL) instead of the *argU/glyU* array (RG), yielding the same result. Cultures of BL21goldDE3 strains containing pACYC-based vectors expressing copies of the indicated *E. coli* tRNA genes and the T7-driven expression vector for *Pfu* polymerase were induced for 2h with 1 mM IPTG. Fifteen μL of whole cell lysate were loaded on 4-20% PAGE gels and stained with Coomassie stain. RL: pACYC with *argU* and *leuW* tRNA genes. RI: *argU* and *ileY* tRNA genes. IL: *ileY* and *leuW* tRNA genes. RIL: *argU, ileY,* and *leuW* tRNA genes.

Paragraph on page 9, lines 20 to 29:

Figure [7] 8 indicates that Pfu DNA polymerase I expression correlates with functional *argU* and *ileY* expression. *Pfu* DNA polymerase I was expressed in BL21goldDE3 strains either containing no tRNA expression vector (-), pACYC-RIL, or two different isolates of the RILP expression vector. Both RILP isolates displayed functional *argU* expression at the same level as observed with pACYC-RIL vector. However, RILP9 displays no functional *ileY* expression, and in RILP16 functional *ileY* expression is diminished when compared to RIL. Functional *ileY* expression was evaluated by rescue of the *ileY*-dependent production of CBP-3xi-Cre. Fifteen μl of the indicated cultures induced at mid-log growth for 2h with 1 mM IPTG were loaded on a 4-20% PAGE gel and the separated proteins were visualized by Coomassie blue staining.

Paragraph at page 10, lines 1 to 7:

Figure [8] 9 demonstrates that the expression of *Pfu*-polymerase II subunits I and II are dependent on functional co-expression of the *argU* and *ileY* genes. *Pfu* DNA polymerase I and the two sub-units of *Pfu*-DNA polymerase II were expressed in the indicated host strains. Fifteen μl of the cultures induced at mid-log growth for 2h with 1 mM IPTG were loaded on a 4-20% PAGE gel, and the separated proteins were visualized by Coomassie blue staining. "-": BL21gold DE3. RG: *argU* and *glyU* tRNA genes. RI: *argU* and *ileY* tRNA genes. IL: *ileY* and *leuW* tRNA genes. RIL: *argU, ileY,* and *leuW* tRNA genes.

Paragraph at page 10, line 23 to page 11, line 6:

This invention is directed toward the problem of rare codon usage in a host cell, which limits expression of a desired recombinant protein. "Codon usage" refers to the frequency with which a given codon appears in the coding regions of a gene. The codon usage of a host cell refers to the average codon usage for known genes which are endogenous to the host cell. A codon or its usage is "rare" if its frequency of use in the host cell is such that depletion of the corresponding tRNA species occurs during expression (particularly high level expression (see below)) of a heterologous protein of interest. A codon which is "rare" in a given host cell may be one which is normally not used by the host cell at all or which is used by the host cell in less than 1% and even less than 0.5% of the host cell genes, or may be one which becomes limiting for the level of expression of a protein of interest. [For example, several rare codons found in different organisms are presented in Tables 1-4.]

Paragraph at page 14, line 20 to page 15, line 8:

Specialized arrays of tRNA genes biased towards the codon usage of specific organisms are utilized according to the invention. [Table 1 lists the codons that are used with a frequency of less than 1% in *E. coli*, and compares the frequency of use in several other organisms.] Frequently used codons (arbitrarily designated as codons with a frequency of more than 1.5%) in heterologous genes constitute potential limitations to heterologous protein expression in *E. coli*. Using [Table 1, or analogous] tables prepared for other host cells, arrays of tRNA genes can be designed to meet the needs of a high level expression system for any organism. For example, one possible array for expression of human proteins in *E. coli* would include the cognate tRNA genes for codons AGG and AGA (Arg); CCC, CCU, and CCA (Pro); GGA and GGG (Gly); and UCC (Ser). Another example of an array that could be selected [from Table 1] is the combination of arginine and proline codons (AGG and AGA (Arg); CCC (Pro)), which could be provided, for example, by the *E. coli* tRNA genes *argU* and *proL*. Yet another example of an array that could be selected [from Table 1] is the combination of arginine, isoleucine, and leucine codons (AGG and AGA (Arg); AUA (Ile); and CUA (Leu)), provided, for example, by the *E. coli* tRNA genes *argU*, *ileY*, and *leuW*. By designing arrays of tRNA genes in this way, it is

possible to tailor an expression host to meet the needs of any desired recombinant protein with a particular codon bias.

Paragraph at page 15, line 24 to page 16, line 4:

In a preferred embodiment of an array according to the invention, which targets the codons AGA or AGG (Arg)), CUA (Leu), AUA (Ile) and CCC (Pro) in *E. coli*, the choice of these codons is based on their infrequent use in *E. coli* [(see Table 1)], and the availability of the cognate tRNA genes. To prepare this embodiment, an array of four tRNA genes which encode tRNAs rarely expressed in *E. coli*, *argU, ileY, leuW and proL* (recognizing the codons AGA/AGG, AUA, CUA and CCC, respectively) was introduced into a low copy number plasmid, pACYC-LIC (see Example 1). These four genes, which are rare in *E. coli*, were isolated from *E. coli* K12. Introduction of this plasmid into suitable protein expression hosts such as *E. coli* BL21DE3 allows high level of expression of proteins normally restricted by the presence of rare codons.

Paragraph at page 27, line 19 to page 28, line 3:

Since genes affected by AUA (ile), CGA (leu) or CCC (pro) were not available, tester constructs were generated based on the observation (Rosenberg, 1993, *supra*) that rare codons affect translation in *E.coli* most when present in a consecutive arrangement at the N-terminus of the protein. Three consecutive leucine (CUA), isoleucine (AUA), or proline (CCC) codons were introduced at the 5' end of the recombinant CBP/Cre fusion gene. Upon IPTG induction, unmodified CBP/Cre fusion protein was expressed at approximately 30% of the total protein, most, if not all of it being soluble. As can be seen in Figure [2] 3, introduction of the rare leucine codon CUA did not affect expression of the recombinant gene. The functional expression of the *leuW* gene could therefore not be assessed. In contrast, introduction of the isoleucine codon strongly reduced expression of the recombinant protein. Expression could be rescued by expression of sets or arrays containing the *ileY* gene, but not by the RG *(argU and* glyU) set, which lacks the *ileY* gene. Thus, the *ileY* gene in the RIL array is functional and specifically rescues expression of genes affected by the rare isoleucine codon AUA.

Paragraph at page 28, lines 4 to 19:

The same strategy was applied to test for functional expression of the *proL* gene in the RILP array. As can be seen in Figure [3] 4, the presence of the RILP array, but not the RIL array, rescued expression of a tester construct containing the cognate CCC codon. Therefore, the *proL* gene in the RILP array is functional. However, although the *argU* gene in the RILP array performs at the same level as observed in the RIL array, functional expression of the *ileY* gene in the RILP array was diminished when compared to the RIL array. Five independent isolates of the pACYC-RILP construct were tested and the same effect was observed in all constructs, albeit to a different extent (ranging from undetectable expression to detectable, but diminished). Sequencing the RILP-tRNA gene arrays revealed that in isolates failing to show *ileY* activity, the *ileY* gene contains a point mutation (A --> T at nt 50 of the mature tRNA) that prevents proper folding of the tRNA and thus is likely to inactivate it. However, in the RILP isolates displaying diminished activity, the sequences of the *argU*, *ileY*, and *leuW* tRNA genes were indistinguishable from the RIL array. This observation suggests a potential incompatibility of simultaneous ectopic expression of the *proL* and *ileY* tRNA genes, possibly due to either interference of the *proL* gene with *ileY* transcription and/or processing or due to an attenuation of the host cells.

Paragraph at page 28, line 22 to page 29, line 7:

In order to test for potential deleterious effects of the RIL and RILP arrays, the expression of four well-expressing T7-driven recombinant genes were compared in BL21goldDE3 cells and their pACYC-RIL and pACYC-RILP containing derivatives. As shown in Fig. [4] 5, no differences were observed between BL21goldDE3 cells and their tRNA-supplemented derivatives in the induced expression level of λ-phosphatase or JNK. However, a loss of expression was observed for calmodulin. The differences between the lines for calmodulin expression were significantly smaller when the induction time was extended, and great variations in induction efficiencies were observed for calmodulin but not for any of the other tested constructs. The reason for the suppression of calmodulin expression is unclear. For chemically competent pACYC-RIL cells, transformation efficiencies of 1×10^8 /μg of pUC18 could be achieved, which was the expected efficiency for a BL21goldDE3 derived cell line. Aside from the induction of calmodulin, no negative effects of the pACYC-based tRNA expression arrays on host cell performance have been detected.--

Paragraph at page 29, lines 10-17:

In order to find further examples of genes aided in their bacterial expression by ectopic tRNA expression, six recombinant *Pyrococcus furiosus* genes were tested that are only expressed poorly in *E. coli*. The choice of archaebacterial genes was based on their strong bias for rare *E. coli* codons, especially AGG/AGA and AUA ([Table 1;] in fact, 95% of the arginine codons are AGG or AGA). Of six tested constructs, four were rescued by co-expression of the pACYC-RIL construct. The enhanced expression of *Pfu*-polymerase I, *Pfu* polymerase II subunit I, *Pfu* polymerase II subunit II, and *Pfu* pyrophosphatase is shown in Figs. [5 and 8] 6 and 9.

Paragraph at page 29, line 21 to page 30, line 11:

Judging by the codon usage of *Pyrococcus furiosus,* genes from this organism are expected to be affected by AGG/AGA (arginine) and AUA (isoleucine) codons. The sequence of *Pfu* DNA polymerase I contains several pairs of rare arginine and isoleucine codons. In order to test the effect of the simultaneous presence of extra copies of the *argU and ileY* genes on expression of *Pfu*-polymerase, IPTG-induced expression level of this gene was compared in strains containing extra copies of different combinations of tRNA genes. As shown in Fig. [5] 6, expression of *Pfu* DNA polymerase I is enhanced by the presence of the *argU* gene in the RG set (*argU* and *glyU*) but to a significantly smaller degree than is achieved by a set or array containing both the *argU* and *ileY* genes (RI and RIL). Thus, simultaneous expression of *argU* and *ileY* yielded about 5-fold higher expression of *Pfu* DNA polymerase I than expression on either gene alone. The set containing only the *ileY* gene but not the *argU* gene (IL) failed to enhance expression of *Pfu* polymerase when compared to BL21goldDE3 cells. However, the *argU* gene in the RG set and the *ileY* gene in the IL set were sufficient to rescue expression of constructs only affected by AGG/AGA codons (hcTnT) or AUA codons (CBP-3xi-Cre) to the same level as was observed with the RI and RIL. The lack of efficient *Pfu* DNA polymerase production in host cells containing the RG set was not due to potentially negative effects of the *glyU* tRNA, because the same effect was observed in cells carrying the RL set (Fig. [6] 7).

Paragraph at page 30, lines 12 to 19:

As described herein, the expression of the *ileY* gene is compromised in different isolates of the RILP array. Two isolates were used (RILP9, lacking detectable functional *ile Y* and RILP

16, displaying diminished *ile Y* function when compared to RIL) to demonstrate the dose-dependence of *Pfu* DNA polymerase expression on *ileY* expression. As shown in Fig. [7] 8 the expression of *Pfu* DNA polymerase in the RIL and RILP strains correlates with the functional expression of *ileY*. Thus, efficient expression of *Pfu* DNA polymerase in *E.coli* is dependent on the simultaneous presence of extra copies of the *argU* as well as the *ileY* gene.

Paragraph at page 30, lines 20 to 28:

To test whether the improved expression of the target gene by simultaneous expression of several different tRNA genes is restricted to *Pfu*-polymerase 1, two additional proteins, *Pfu* polymerase II subunit I and subunit II were tested. Judged by their sequence, both genes should be similarly affected. In fact, expression of both genes was enhanced about 5-fold by coexpression of the *argU and glyU* tRNA genes (Fig. [8] 9), similar to the observation with *Pfu* DNA polymerase 1. This suggests that the requirement for extra copies of two or more different tRNA genes for efficient gene expression in *E.coli* may be a frequent phenomenon for genes derived from organisms possessing a distinct codon usage from that of *E. coli*.

At page 7, after line 21, insert the following:

Figure 2 displays a Coomassie blue-stained SDS PAGE gel containing samples from cells containing a T7-driven expression vector for wild type human cardiac troponin T (hcTnt wt; *argU*-dependent; Hu et al.) and two mutants thereof (mut1, mut2), before and after IPTG treatment and in the presence and absence of an expression vector for the tRNA genes *argU* and *glyU* (RG). The position of the TnT protein is shown with an arrow.